

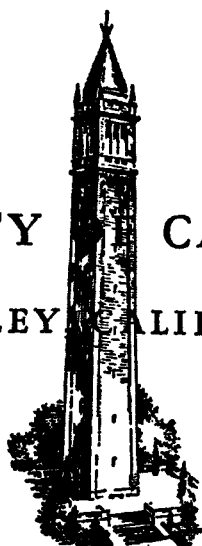
SPACE SCIENCES LABORATORY

FACILITY FORM 602

| | |
|-------------------------------|------------|
| N65-20625 | |
| (ACCESSION NUMBER) | |
| 65 | THRU |
| (PAGES) | |
| 013-57585 | (CODE) |
| (NASA CR OR TMX OR AD NUMBER) | 11 |
| | (CATEGORY) |



UNIVERSITY CALIFORNIA
BERKELEY CALIFORNIA



GPO PRICE \$ _____

OTS PRICE(S) \$ _____

Hard copy (HC) \$3.00

Microfiche (MF) .75

Space Sciences Laboratory
University of California
Berkeley, California 94720

Semiannual Progress Report on
ENZYME ACTIVITY IN TERRESTRIAL SOIL IN
RELATION TO EXPLORATION OF THE MARTIAN SURFACE

NASA Grant NsG 704

Series No. 6

Issue No. 8

Department of Soils and Plant Nutrition
Period 1 July 1964 - 1 February 1965

March 9, 1965

OUTLINE of REPORT

- A. Statement of the Problem.
- B. A Survey of the Biochemistry of Terrestrial Soils
(submitted for publication to National Academy - NASA
Summer Study Report).
- C. A Brief Survey of Enzymes Found in Terrestrial Soils.
- D. Urease Activity in Media of Low Water Content.

By J. O. Falkinham

J. Skujins

A. D. McLaren

A. Statement of the Problem

In "A Review of Space Research," National Academy of Sciences Publication 1079, pg. 9, it is stated that NASA endorses the current development of a life-detection experiment based on enzyme activity. We in this department have been studying enzyme activity in soil, and in soil which has been sterilized by ionizing radiation, for a number of years and we would like to try to make a contribution to the program.

Terrestrial soil is a living tissue. It contains, in addition to clay minerals, sands, water, air, organic matter, and inorganic salts, a host of microorganisms. These microorganisms, the fungi, bacteria, algae, etc., secrete or liberate upon death an unknown number of enzymes. It is not clear at present whether the extracellular enzymes are important in plant nutrition, but the amount of soil enzyme activity may actually be greater than that of the living components of soil at any one time (G. Hoffmann, personal communication). As yet only urease has been extracted from soil, but other enzyme activities in soil are easily demonstrated (1). Much of this activity may be associated with the clay fraction (2); certainly enzymes adsorbed on clays are stabilized from attack by microorganisms and retain enzyme activity in the adsorbed state (1,3,4).

One way of demonstrating enzyme activity in soil is to arrest microbial metabolism by adding toluene followed by addition of a suitable substrate for the enzyme in question. We have, however, developed a method of differential sterilization of soil using

ionizing radiation. By increasing the dose one can kill first fungi, then actinomycetes and bacteria. Enzyme activity remains after doses of 2 to 5 million rep. Enzyme activities tested for and found include urease, protease, phosphatase, esterase, and respiratory enzymes (5-9).

During these studies it occurred to us that if life ever existed on other planets now devoid of life, residual enzyme activity might be found there by soil analysis (7). Further, if life is present in Martian and other extraterrestrial soils, enzyme tests applicable to terrestrial soil might be suitable. It is the object of this proposal to develop qualitative tests for the various enzyme activities in soil and to adapt the most sensitive of these to procedures by which identifying information can be transmitted by radio from the Moon and Mars. Obviously we should learn much more about earth soils with this program.

Approach

Three overlapping possibilities in connection with the above.

- a) Extra~~ter~~restrial life is a function of an unfamiliar biochemistry.
- b) Life may now be extinct on Mars.
- c) Life, with a familiar biochemistry is extant on Martian soil.

If life is now extinct on Mars, evidence of life which might be "recognized" by equipment in a Rocket on Martian soil could be gleaned from residual soil enzyme activity. We have observed (7) that soil phosphatase in soil has about the same sensitivity to ionizing

radiation as purified potato phosphatase in a dry state. Thus residual soil enzymes would be subject to inactivation by surface radiation on Mars at a rate depending on depth beneath the surface, radiation intensity, radiation energy, and hydrolytic and oxidative reactions. Radiation inactivation of soil phosphatase follows the equation

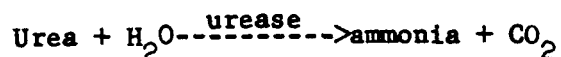
$$\ln A = \ln A_0 - KIt$$

where A is the residual activity after a time t of irradiation at an intensity I reaching the soil enzyme. A_0 and K are constants, A_0 being the activity at the cessation of life. Unfortunately we would not know both t and A_0 and could not calculate one from the other. On earth, however, we know that enzymes may be found at depths of from several inches to feet in soil. Thus, special radiation energies may not be great enough to reach sub-surface enzymes on Martian soil. Nevertheless, if b is true, the rocket equipment would need to sample beneath the surface before one could conclude that Mars has always been sterile.

Tests for soil enzymes can involve several tricks or techniques. For examples:

1. Formation of a volatile product.
2. Formation of a fluorescent product.
3. Formation of a light absorbing product or one which can be converted to a colored compound.

A typical soil reaction for 1. is the following (5):



Both products could be labelled with radioactive isotopes for sensitive analysis.

In Professor Lederberg's Laboratory success has been achieved in preparing fluorescent derivatives of fluorescein, such as fluorescein diphosphate. Upon catalysis by phosphatase, fluorescein is released; it can be measured by fluorescence excitation. Another type reaction which should be explored is the following:

Beta naphthol + N-dimethylaminoaniline $\xrightarrow[\text{(O)}]{\text{peroxidase}}$ Meldola blue
(an oxasine dye).

The product of peroxidase reaction here is also a member of a family of fluorescent dyes.

An example of the third type of reaction would be the action of an esterase on phenylacetate, and peptidase action:

Benzoylarginine-paranitroanilide + H₂O $\xrightarrow{\text{trypsin type protease}}$
paranitroaniline etc.

Here the amount of product could be measured by differential spectrophotometry at a suitable wavelength absorbed selectively by the product. Since soil contains appreciable amounts of organic materials which may also absorb light and fluoresce differential absorption or emission equipment may be required in analysis.

In all these studies the apparent amount of an enzyme in soil should be measured in terms of the activity of purified enzymes from microbial sources. Further efforts should be expended on methods of extracting enzymes from soil.

Since the humidity on Mars is so very low, the virtually unexplored subject of enzyme activity in systems of low water content should be investigated.

References

1. Symposium on Soil Enzymes, VIII International Congress on Microbiology, Montreal, 1962.
2. A. Haig, Ph.D. thesis, University of California, Davis, 1955.
3. E. F. Estermann, G. H. Peterson and A. D. McLaren, Digestion of Clay-Protein, Lignin-Protein, and Silica-Protein Complexes by Enzymes and Bacteria, Soil Sci. Soc. Am. Proc. 23, 31 (1959).
4. E. F. Estermann and A. D. McLaren, Stimulation of Bacterial Proteolysis by Adsorbents. J. Soil Sci. 10, 64 (1959).
5. A. D. McLaren, L. Reshetko and W. Huser, Sterilization of Soil by Irradiation with an Electron Beam, and Some Observations on Soil Enzyme Activity. Soil Sci. 83, 497 (1957).
6. A. D. McLaren, R. A. Luse and J. J. Skujins, Sterilization of Soil by Irradiation and Some Further Observations on Soil Enzyme Activity. Soil Sci. Soc. Am. Proc. 26, 371 (1962).
7. J. J. Skujins, L. Braal and A. D. McLaren, Characterization of Phosphatase in a terrestrial soil sterilized with an Electron Beam. Enzymologia, 25, 125 (1962).
8. G. H. Peterson, Respiration of Soil Sterilized by Ionizing Radiations. Soil Sci. 94, 71 (1962).
9. G. H. Peterson, Microbial Activity in Heat and Electron-Sterilized Soils Seeded with Micro-organisms. Canadian J. Microbiology 8, 519 (1962).

B. A Survey of the Biochemistry of Terrestrial Soils

Introduction

The Survey

1. General description of soil biochemistry
2. Some kinds of organic substances in soil
 - a) Enzymes
 - b) organic nitrogen compounds
 - c) organic sulfur compounds
 - d) organic phosphorus compounds
 - e) sugars
 - f) humus
3. Remarks concerning tests for evidence of life in soil

INTRODUCTION

In testing for life or for evidence for life processes in soil one can use either nutrient media in which organisms can grow or else choose reagents which will react specifically with products characteristically produced by living entities. The former presupposes either universal medium or a medium in which some specific, universally dispersed soil organism will proliferate. This type of test may fail in Martian soils if an unfamiliar biochemistry prevails. With the latter

approach one must distinguish between compounds similar to those found in Miller-Urey experiments and those more characteristic of life, such as enzymes and nucleic acids. These are usually monodisperse with regard to molecular weight and possess non-random structures. All of these exist in nearly undetectable amounts in Earth soils, since they are nutrients for at least some of the organisms present. On the other hand, a few of the soil organic molecules, the enzymes, may be revealed by virtue of their ability to "turn over" appreciable amounts of substrate. Here we make the assumption that although the nutritional requirements of organisms in strange soils may be unknown, the enzyme constituents released to the environment by decomposed organisms are universal in general properties and characteristic of life wherever it is found.

Obviously life forms on Mars and elsewhere may differ too much from those here to be tested for within this framework. This report, however, will only presuppose that the following discussion may suggest something concrete and accessible in the way of "life testers."

THE SURVEY

1) General description of soil biochemistry.

Earth soils are aggregates of minerals, water, humus and microorganisms. As a kind of a graveyard of microorganisms, a soil may be expected to contain almost any naturally occurring organic compound (1). The relative abundance of these extracellular compounds is far from that found in living tissues, however. Additional substances, perhaps characteristic of soil per se, are also present in abundance.

From one point of view the microbiologist is confronted with soil as a unique medium, in which organisms live, not in pure culture but in highly complex populations comprising innumerable species; these exert a variety of associative and antagonistic effects (2). There seem to be countless numbers of different "species" of bacteria, yeasts, protozoa, fungi as well as microfauna in soil. In assaying for unique biochemical activities, certain groups of these have been found to be geared to elemental cycles such as ammonification, nitrification and nitrogen fixation. From a somewhat different point of view, Quastel (3) adopted another conceptual scheme, namely that the soil as a whole be considered as an organ, comparable in some respects to a liver or a gland, to which may be added various nutrients, pure or complex such as degraded plant materials, together with rain and air and in which enzymatic reactions can occur. The products of these reactions are important as steps in elemental cycles in the percolation (movement) of iron and aluminum, as humates, and in the formation of soil crumb structure. The notion here is that the soil biochemist is more concerned with what the microbes are doing in soil rather than in precisely what they are with respect to size, shape, or other ingredients which make up taxonomic schemes.

In the form of enrichment cultures these two approaches come together. If glycine is perfused repeatedly through a sample of native soil, together with the products of microbial activity, those organisms capable of metabolizing glycine, ammonia and nitrite will increase greatly in numbers, and isolates of these, for example Nitrosomonas sp. can be more easily prepared. In this example certain

cells of the soil-organ are encouraged to multiply and to "cooperate" in the conversion of glycine to nitrate, carbon dioxide and water as principal products. Along with these reactions, however, and in the presence of carbohydrate, other soil organisms can synthesize anionic, high polymeric substances, some of which are absorbed on the clays. A kind of a steady state exists at any interval in time, depending on the rates of addition and loss of metabolites to and from the soil.

In the following is described some of what is known about biochemical reactions in soil and the nature of soil organic matter. It is not clear how well, or poorly, this information will be useful in anticipating the bio-organic chemistry of Martian soils, if indeed the surface of Mars has any areas comparable to Earth soils. It may, however, lead to some guesses as to what one should look for on Mars if life as we know it has some counterpart there.

Ecological Conditions in Soil. First of all, let us consider the environment in which the soil microorganisms dwell. It differs rather drastically from that of a culture flask or an agar slant. There is a point to point variation in concentration of all solutes, interspersed in a matrix of clays, sand and humus which characterizes a spectrum of microenvironments. Further, at the surfaces of these particles as well as at plant roots there is a variation in molecular environment characterized by gradation in pH and redox potential (4,5).

Morphologists have dissected soil into three principal horizons, namely A) the upper or alluvial layer which receives litter and from which material is being, or has been, leached; B) the underlying

illuvial layer which is being, or has been, enriched; and C) the mineral, parent material underneath A and B horizons. These horizons may be well delineated in profile or the B horizon may arbitrarily grade into the other two. Pore space occupies 30 to 45 percent of the soil and varies in water and air content. From A to C the total numbers of microbes per gram of soil decline and the ratio of aerobes to anaerobes declines. Soils with greater humus and/or clay content tend to hold more water, because of hydration and the presence of capillary pore space associated with small particle size, than do soils with the larger, silt particles. In "waterlogged" soils there are practically no air spaces, and, in "air dry" soils, the pore liquid water has almost disappeared leaving only water films about the particles.

Microorganisms cannot grow in the absence of water. The lower limits of relative humidity at which growth is possible depends upon the species. Their activities may depend on the thickness of the water film; for example, Rahn (6) found the optimum film thickness to be 20 to 40 μ for Bac. mycoides, an aerobe. Some bacteria can be stored under dry nitrogen, without growth, however. Winogradsky determined depths to which an aerobe, *Azotobacter*, and an anaerobe, *Clostridium*, could grow in wet soils: at a moisture content of 23% the former were limited to the extreme surface and the latter were found throughout the soil (7).

Recently from a study of widely different soils it has been found that "changeover" from aerobic to anaerobic metabolism of organic materials takes place in widely different soils at an oxygen concentration less than about 3×10^{-6} M, a very low concentration indeed (8).

Decomposition below this concentration in soil leads to the accumulation of fatty acids. One implication is that water-saturated crumbs of a soil greater than about 3 mm in radius have no oxygen at their centers, and since crumbs of this size are present in most soils it means that pockets with anaerobic conditions are ubiquitous, and this provides an explanation for the universality of strict anaerobes. Oxidative processes abound in relatively warm, dry soils. Contrawise, in relatively wet soils organic matter content increases with increasing rainfall (9).

Waterlogged soils, high in organic matter, tend to become acidic due to fermentation, whereas well aerated garden soils are near neutrality, i.e. the pH is of the order of 6 to 7.5 as measured with wet pastes and a glass electrode system. At higher pH, as in calcareous soils, growing plants may show Fe or Mn deficiencies because of the insolubility of the corresponding phosphates and carbonates. The addition of sulphur results in the lowering of pH and the liberation of these cations as will be discussed below.

Upon examining the effective pH at the surface of soil colloids, however, a new concept emerges. Wherever charged surfaces exist in contact with water, the effective pH of the surface (pH_s) will be lower than the pH in bulk (pH_b). This difference has been expressed by Hartley and Roe as $pH_s = pH_b + \frac{\delta}{160}$ at 25° where δ is the electrokinetic potential of the particle (10). In fact, chymotrypsin acting on a protein adsorbed on kaolin has a different pH optimum for enzyme action than is found in solutions with the same substrate for this reason. The optimum pH of succinate oxidation with cells of E. coli adsorbed

on an anion exchange resin differs from that of free cells suspended in solution and a study of the initial velocities of oxidation of this substrate with free and adsorbed cells at $\text{pH}_b = 7$ revealed that the velocity reached a maximum at a ten times higher concentration with adsorbed cells than with free cells (11). These observations show that the heterogeneity of soil as an environment for microorganisms extends from the gross particle to the molecular level.

It is recognized that in a "living" soil the elements are continuously subjected to biochemical transformation and to both biological, mechanical, and hydrological translocations.

Some Metabolic Processes in Soil. The oxidation of ammonia or of sulfur in soil has been studied with a biochemical technique of broad application. A nutrient solution is continuously perfused repeatedly through a column containing about 30 grams of soil in a water saturated, aerated condition (12). The technique is splendid for characterizing nitrification in soil nitrifying organisms until a "saturation" of numbers exists and that the conversion $\text{NH}_4^+ \longrightarrow \text{NO}_2^-$ took place largely on particulate surfaces. In another study, sulfur was added to a sample of Fresno fine sandy loam (a black alkali soil initially of pH 10.2 and a salt concentration of less than 0.2%) to the extent of 1g/30g soil (13). The mixture was perfused with 200 ml water and the perfusate was analysed for hydrogen ion and sulfate ions as a function of time. After a few days of perfusion, sulfate was found in the perfusate and the pH fell from the over-all reaction $1 \frac{1}{2} \text{O}_2 + \text{S} + \text{H}_2\text{O} \longrightarrow \text{H}_2\text{SO}_4$. The addition of Thiobacillus thiooxidans to the soil did not greatly

modify the rate of the reaction, showing that related organisms existed in the field soil initially. After 20 days of perfusion the soil was washed free of sulfate and reperfused; oxidation took place at the maximum rate. Clearly at this point the soil is enriched with respect to sulfur oxidizers.

Sulfide and ammonia are reduction products of decompositions of some common organic compounds; both may be oxidized by certain soil microbes and a few autotrophs can use these oxidations as sole sources of energy (14). In a soil perfused under aerobic conditions, and containing an indigenous population, practically all sulfur in cystine becomes sulfate and the corresponding nitrogen becomes nitrate. The acidity of the soil increases and other organisms may be inhibited. A single sulfur compound, however, may yield different sulfur products on dissimilation by different microbes. From cystine, Achromobacter cystinovorum produces only elemental sulfur.

The following pathway must be considered as a logical over-all process for the soil as an organ but not meant to pertain to any particular organism: cysteine \longrightarrow cystine \longrightarrow cystine disulfoxide \longrightarrow cysteine sulfinic acid \longrightarrow cysteic acid \longrightarrow sulfate (14).

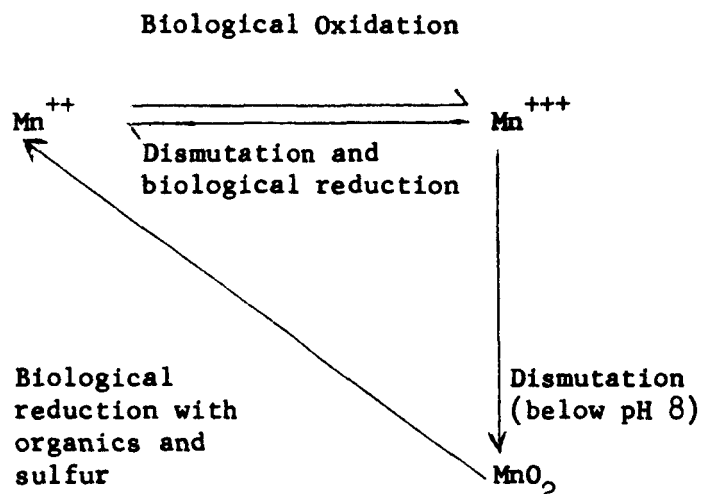
In all such experiments it must be remembered that the amount of free cysteine normally in soil is minute and one of the great difficulties is to decide if the reactions observed are typical of the microbial population under field conditions or only when the system is jammed with substrate. An abundance of cysteine changes the redox potential of soil and also in other ways may lead to an enrichment of only a small proportion of the qualitative makeup of microbes present. Nevertheless,

in some areas of the world, for example New England, Australia, most of the soil sulfur is locked up in some form of organic matter and pasture lands in this region are, at present, mainly dependent for nutrition on a release of this sulfur by mineralization. From solubility studies the organic sulfur appears to be in the form of high molecular fulvic acid sulfates (see 2.f, below). Here, an element has been taken out of circulation by the formation of macromolecules. A similar story pertains to nitrogen.

Perhaps it is needless to say that in the organ approach to soil we have no way of deciding which reactions may be attributed to a given species. Historically, one has studied isolates of single species, but in doing so, one misses the possible interactions among species, with mutual inhibitions and stimulations. This conceptual difficulty is not unique to soil as a tissue, however. A good illustration involves manganese metabolism. Bromfield and Skerman found that manganese sulfate could be oxidized with the formation of a brown deposit of MnO_2 on agar plates if a certain two species were growing as colonies near each other. Two such pairs had Corynebacterium in common; the other organism could be either a Flavobacterium or a Chromobacterium. Obviously the existence of associative action between microbes makes it virtually impossible to count the number of Mn^{++} oxidizing organisms in soil (16). An ascomycete, Cladosporium, could oxidize this ion without association.

If manganese sulfate is perfused through a neutral or slightly alkaline soil, it is found that after a few days all the Mn^{++} has disappeared from the perfusate. Above 0.02 M the rate of formation of


MnO_2 falls rapidly, indicating manganese toxicity. Also metabolic poisons including azide and iodoacetate bring about a marked inhibition of the oxidation. Detailed observations have been summarized as follows (17):



Reduction of manganese oxides in soils can be brought about with added glucose, thiols and polyphenols. These laboratory observations provide a useful explanation for the fact that applications of manganese sulfate to deficient field soils are often ineffective as MnO_2 represents an unavailable form of the element. An increase in concentration of available manganese can be brought about by the addition of reducing forms of organic matter, reduction of pH so as to shift the "cycle" toward Mn^{++} , inhibition of oxidation by specific poisons and stimulation of manganese reducing organisms. Here one can "over-lap" the sulfur and manganese cycles to advantage: the addition of sulfur favors the reduced form of manganese by formation of sulfuric acid, with a reduction in pH, and by slow thiosulfate production. The addition of sulfur to a "manganese deficient" soil may exert a beneficial effect on the growth of plants (17).

Generally, if appreciable amounts of an organic nutrient such as glucose is added to soil, the results are profound and in many directions. We mention phosphate accessibility as another example. Sperber has found that certain fungi, actinomycetes and bacteria can solubilize apatite ($\text{Ca}_3(\text{PO}_4)_2\text{CaF}_2$) by virtue of lactic, glycollic, citric and succinic acid production. Numerous have been the reports that soil microorganisms increase the availability of phosphates to plants (18).

Soil Organic Matter and Soil Structure. The addition of glucose to soil can have important physical consequences as well. Certain of the bacteria synthesize large amounts of polysaccharides as by-products or storage materials. Some of the added glucose goes into protein formation, thus removing soluble nitrogen from the soil solution (rendering nitrogen temporarily unavailable for plants). The polysaccharides, particularly those with carboxyl groups become adsorbed to the soil colloids.

Organic matter in soil plays an essential part in securing the structure that is required for high fertility. From synthesis at the expense of substances of plant origin and from autolysis of microorganisms, macromolecules appear among clay particles which have the capacity to bind to the latter, presumably through, e.g., $\text{R-CO}_2 - \text{Ca} - \text{clay}$ bonds, according to  where the lines represent macromolecular chains and the circles depict negatively charged clay particles. Bacterial gums, alginic acid, pectic acid and a large class of synthetic compounds also have this capacity.

The net effect achieved upon adding such substances to soils high

in clay and low in organic matter are improved aeration, tilth, water percolation rate, and sometimes crop yield, all resulting from the formation of a more stable crumb structure and an increase in stability against compaction (19).

Enzyme Action in Soil. Thus far we have seen some implications of soil biochemical activities in the fields of soil chemistry, physics and fertility. In these matters the microbe has been an active contributor. A number of investigators, however, have asked the question how much of enzyme action in soil results from this activity at any point in time and how much is attributed to an accumulated "back-ground noise" of enzyme activity in soil per se, i.e. to enzymes adsorbed on clays or humus. One approach is to add toluene to soil to suppress microbial activity. Although toluene and even gasoline are substrates for some organisms, in short time experiments life activities may be considered suppressed. Similar results are obtained if soil is first sterilized with an electron beam (20). With a Dublin clay loam, over half of the glycerolphosphatase activity was found after complete sterilization with a dose of 5 Mrep. It will be of significance to know if this residual enzyme activity is outside non-viable cells and how much phosphatase activity in dead cells incapable of acting on substrate added to the sterile soil (21).

Haig (22) has fractionated soils and found that most of soil esterase activity is in the clay fraction and an intriguing question is how is the enzyme held by the clay, how is it accumulated, and how

can it be isolated for study.

An interesting and important problem of soil enzyme action requires a knowledge of the kinetics of enzyme action at solid-liquid interfaces. The phenomenon just described involves the action of an adsorbed enzyme on a soluble substrate. Conversely, microbes secrete soluble enzymes which act on insoluble substrates such as cellulosic debris, soil organic matter, chitin, etc. The kinetics of these reactions are not represented solely by equations of the Michaelis-Menten type for classical reactions in solution. Instead the limiting rates of reaction involve diffusion of substrates to surfaces, adsorption of enzymes on particles and the local pH_s discussed above. Equations for these situations are now available for evaluation (23). The role of water and the minimum activities of water at which enzyme action can take place has yet to be evaluated.

Soil Development. Studies of the microbiology of soil development has only begun. Sand-dune communities were chosen by Webley et al. because the development of a soil microflora could be traced from the simple conditions of early colonization of bare sand through a series of communities of increasing botanical complexity accompanied by the formation of a soil (24).

Microbial counts were obtained from a transect across a dune at Newburgh (Aberdeenshire). In progressing from open sand past early fixed dunes to dune pastures and heath, the number of bacteria rise from a few thousand to millions per gram as soon as vegetation colonizes

the sand. There is a fall in numbers of bacteria, but not of fungi, as heather, with an acid humus, enters as a dominant plant. In comparing rhizosphere soil with nearby soil there is a marked increase in microbes on passing from open sand to root surfaces of Agropyron and Ammophilia, with predominance of Corynebacteria, Mycobacteria, and Nocardia among the "bacteria" throughout. Among the fungi, however, Penicillium sp. predominated in open sand and Cephalosporium near roots. Each plant species develops a unique rhizosphere flora which overlaps only in part other species. There can be little doubt that the activity of the microbes contributes to the development of the soil and the maturation of the habitat in a reciprocating way with higher plants growing from a sand and salt milieu.

The concept of a development of a soil profile, involving the leaching and translocation of aluminum, iron, humic acids and other chelating agents, etc., together with co-precipitation, is one which will require some long-time experiments. The evidence is presumptive and ad hoc as in evolutionary theory generally, but no one seems to question seriously the over-all picture.

2) Some kinds of organic substances in soil.

a) Soil enzymes.

The bulk of organic substances in Earth soils undoubtedly arise from the decomposition of animal and vegetable residues by microorganisms. This is not to say as a liberation by autolysed cells but rather as a cycling of the elements via first the digestive and then the synthetic

activities of soil flora and fauna. The microfauna are very important in the A horizon where at leaf litter is degraded in the presence of abundant oxygen, particularly in acid, forest soils. Lower down, in the soil, the flora tend to finish the catabolism begun by microfauna and all recognizable vestages of plant cytological structure vanish (25). These degradations release enzymes, the exoenzymes of digestion. Upon starvation, microflora and fauna may autolyse with release of intracellular enzymes. These details have not been worked out. In Table I Briggs and Spedding (26) have listed some enzymes found in soil. We know nothing of the abundance of these enzymes in soil and whether they may be found both in and outside soil microbes. Soil phosphatases are of several kinds, e.g. glycerol phosphatase and phytase. The latter is very important in the soil phosphorus cycle. About 60 percent of soil phosphorus is typically organic but only a small fraction of this is of known structure, namely as in phytin.

Of all the soil organic substances, individual enzymes are most easily tested for. Although only small amounts are present, the turnover of substrate, particularly in the cases where the products are volatile or fluorescent is easily measured quantitatively. In fact, B. Rotman has developed a test for one or more enzymes which is sensitive at the one-enzyme-molecule level. Clearly there cannot be much free enzyme-protein in soil as proteins are nutrients for organisms, but enzymes may be chemically cross-linked with humus. It is known that such bound enzymes may still function (27).

It is also of interest that soil sterilized by ionizing radiation can still respire, but at a reduced rate, indicating the presence of

Table I
Some Soil Enzymes
(From Briggs and Spedding, 1963)

| Name of Enzyme | Reaction Catalysed |
|------------------------------------|--|
| 1. Urease | Urea \longrightarrow ammonia + carbon dioxide |
| 2. Amylases | Starches \longrightarrow sugars |
| 3. Glycosidases | Glycosides \longrightarrow sugars + aglycones |
| 4. Asparaginase | Asparagine \longrightarrow aspartate + ammonia |
| 5. Aspartate-alanine transaminase | Aspartate + pyruvate \rightleftharpoons alanine + oxalacetate |
| 6. Catalase | $2\text{H}_2\text{O}_2 \longrightarrow 2\text{H}_2\text{O} + \text{O}_2$ |
| 7. Invertase | Sucrose \longrightarrow glucose + fructose |
| 8. Proteases | Proteins \longrightarrow peptides |
| 9. Dehydrogenases | Reduced substrate \longrightarrow oxidised substrate |
| 10. Glutamate-alanine transaminase | Glutamate + pyruvate \rightleftharpoons alanine + α -ketoglutarate |
| 11. Glycerophosphatase | Glycerol phosphate \longrightarrow glycerol + phosphate |
| 12. Inulase | Inulin \longrightarrow fructose and fructose oligo-saccharides |
| 13. Leucine-alanine transaminase | Leucine + pyruvate \rightleftharpoons alanine + α -ketoisovalerate |
| 14. Nuclease | Purines, etc. \longrightarrow ammonia + keto-purines (etc.) |
| 15. Peroxidase | Substrate + $\text{H}_2\text{O}_2 \longrightarrow$ oxidised substrate + H_2O |
| 16. Phosphatases | Organic phosphates \longrightarrow compound + orthophosphate |
| 17. Polyphenol oxidase | Polyphenols + $\text{O}_2 \longrightarrow$ quinones + H_2O |
| 18. Tyrosinase | Tyrosine + $\text{O}_2 \longrightarrow$ o-quinones + H_2O |

Table II
Nitrogen Compounds in Soil

| Substance Tested for | Conditions | N-Content of Soils Tested | Fraction of N as Substance Tested For | References |
|----------------------|----------------------------------|---------------------------|---------------------------------------|-----------------------|
| Amino sugars | After acid hydrolysis | 0.17-2.8 % | 0.05-0.1 | Bremner and Shaw (29) |
| Amino acids | After acid hydrolysis | --- | 1/3 - 1/2 | Bremner (30) |
| Amino acids, free | NH ₄ -O-Ac extraction | --- | 0.001-0.0001 | Paul and Schmidt (32) |
| Protein | Native soil | --- | nil | Van Driel (31) |

some residual organized enzyme systems (28).

b) Organic nitrogen compounds in soil.

Among the recognizable organic nitrogen compounds in soil are amino sugars, amino acids and nucleotides, Table II.

Amino sugars such as glucosamine are derived from chitin. It is clear from the table that spectroscopic examination of soil for peptide bonds or amino acids without suitable extraction from soil humus is out of the question. Some infrared spectra on soil organic matter have been reported; the evidence for soil protein is almost nil (33). Further, the amino acids derived from soil organic matter following hydrolysis are not of protein origin. The liberated amino acids may have been in condensation products of phenols or quinones (25). Free amino acids may range from 2-4 mg per Kg soil; in soil the poly-basic amino acids are adsorbed to the clays; tryptophan is not (36).

c) Organic sulfur compounds in soil.

At present we can only conclude that there are some organic sulfur compounds in soil, in fact in some Australian soils most of the soil sulfur may be organic (34). Some of it is, of course, in amino acids (Table II).

d) Organic phosphorus compounds in soil.

As already mentioned, most of the soil phosphorus is organic (37). Very little nucleic acids exist in soil (1 ppm), perhaps no more than in the microbes themselves. In one such test the total organic P ranged from 300-500 ppm (35). Several forms of inositolhexaphosphate are found in soil (38); they occur from 100 to 300 ppm (39).

e) Carbohydrates.

Sugars added to soil are quickly metabolized and free sugars are absent in natural soil. On the other hand, polysaccharides, of microbial origin are present in soil (48), to the extent of 0.1% (49). These contain galactose, glucose, mannose, orabinose, etc.

f) Humus.

The over-all chemistry of the major, and insoluble, portion of soil organic matter is called humus. About 1/3 of this organic matter can be removed from soil by extraction with alkali. The extraction is improved by prior treatment of the soil with chelating agents, since humus seems to be held to clay by bonds, involving di- and tri-valent metals. Alternately, treatment of soil with HF removes sand and solubilizes clay minerals, thereby leaving the organic matter. Following neutralization of alkaline extracts, humic acid is precipitated and polymeric, fulvic acid remains in solution. These composite acidic substances contain chemically bound N, P and S. Of course the solution phase also contains the micromolecular compounds described under b, c, and d, and polyuronic acids. Humus may be found in low amounts, ca 1% in desert soils and up to 80% in so-called organic soils.

About one-half of the N in fulvic acid consists of compounds which are deaminated upon hydrolysis; about one-fourth consists of amino acid precursors and one-tenth of amino sugars (40).

Humic acid from one source has been found to contain about 7% methoxyl groups (41), Table III, along with a number of other functional groups. In Table IV a summary is presented of infrared absorbances of organic matter from A and B horizons (45).

Table III
Functional Groups Found in Humus

| Group | Amount | Spectral Absorption | References |
|------------------------------|------------------------|---------------------|------------|
| OH | 2-3 meq per g. | 3.0 μ | 41,45 |
| CH ₃ | | 3.45 | 41 |
| Methoxyl | 6.7%, 0.2-0.4 meq/g | 6.3 | 45 |
| Carbonyl | 4-6 meq per g | 5.85, 6.22 | 41,45 |
| Carboxyl | 2-9 meq per g | --- | 45 |
| Unknown | --- | 7.2 | 41 |
| Aromatic | 27% | --- | 46 |
| Clay impurity | --- | 9.0 | 41 |
| Stable free radicals | 10 ¹⁸ per g | (EPR) | 47 |
| Quinone | --- | --- | 42 |
| Phenolic | 3 meq per g | --- | 45 |
| (e.g. <u>epi</u> - catechin) | | | 43 |
| Unknown | --- | (fluorescence) | 44 |

Table IV
Major Bands and Relative Absorbances in the Infra-red of
Extracted Organic Matter (45)

| Frequency (cm. ⁻¹) | Relative Absorbance | | Interpretation |
|-----------------------------------|----------------------------------|----------------------------------|---|
| | A ^o Organic Matter | B ^h Organic Matter | |
| 3,380 | strong | strong | hydrogen bonded — OH and bonded — NH groups |
| 2,910 | medium | shoulder | aliphatic C—H stretching vibrations |
| 2,840 | medium | 0 | aliphatic C—H stretching vibrations |
| 2,600 | shoulder | shoulder | carboxyl C—H stretching vibrations |
| 1,720 | shoulder | strong | carboxylic carbonyl |
| 1,620 | strong | strong | joint interaction of hydroxyl and carboxyl with carbonyl, also possibly carboxylic groups associated with metals so as to give the carboxylate structure |
| 1,450 | strong | 0 | CH ₃ and/or CH ₂ in plane deformation vibrations |
| 1,400 | 0 | medium | carboxylate |
| 1,250 | weak | 0 | phenoxy C—O |
| 1,200 | 0 | medium | carboxyl |
| 1,030 | medium | 0 | Si—O of silica due to the presence of clay |

3) Remarks Concerning Tests for Life in Soil.

This problem can be divided into two parts, namely the test for living creatures and testing for products of metabolism. The latter products must be distinguished from chemicals formed under the action of other natural agents, such as radiation (for a review see McLaren and Shugar [50]). The former products, as we have tabulated, include enzymes, polysaccharides, and also include certain aromatic substances of unknown structure in a percentage perhaps higher in humus than found in products formed by radiation.

Test methods for enzymes in soil are already under development, for example, phosphatase in the Multivator. As yet we do not know about the relative abundance of enzymes in soil and this should be actively explored. For example, urease is prevalent and urea containing radioactive carbon should provide a sensitive assay comparable to that for phosphatase in the Multivator.

A difficulty with Gulliver, and the Wolff trap, is that we do not know what universal substrate system can be supplied to the Martian "soil" and a test for autotrophs may require growth under light. On the other hand, all microbes contain some enzymes in common and a battery of enzyme assays may be more likely to succeed as a presumptive test for evidence of life.

A third approach ties in with the observation that, following the air-drying of soil, upon addition of water to a soil there is a burst of biological activity and a liberation of CO_2 (51). One would expect that this metabolic activity would result in the liberation of heat and the heat could be measured calorimetrically (52).

A microbiological fractionation of isotopes might also be useful in life detection; e.g., fractionation of sulfur by Desulfovibrio desulfuricans is rather dramatic (53).

Finally, since living things generally contain free radicals, and humus contains trapped free radicals, probably of the semquinone and quihydron type, the finding of free radicals in Martian soil could be taken as presumptive evidence.

Offhand, both the calorimetric and free radical measurements appear to be potentially very important; they are non-specific in the sense that no particular form of life need be probed for, i.e. furnished with a usable medium for growth. Glenn Polloch of N.A.S.A. at Ames is using the new spinco calorimeter with soil with this thought in mind.

References and Notes

1. S. A. Waksman, "Humus," Second Ed. Williams and Wilkins Company, Baltimore, Md., 1938.
2. S. A. Waksman, "Soil Microbiology as a field of soil science," Science 102, 339 (1945).
3. J. H. Quastel, "Soil Metabolism," The Royal Institute of Chemistry of Great Britain and Ireland, London, 1946.
4. K. V. Thimann, "The Life of Bacteria," MacMillan Co., N. Y., 1955.
5. W. L. Kubiena, "Micropedology," Collegiate Press Inc., Ames, 1938.
6. O. Rahn, Zentr. Bakt. II Abt. 38, 484 (1913).
7. S. Winogradsky, Compt. rend. Acad. Sci. 179, 861 (1924).
8. D. J. Greenwood, Plant and Soil 14, 360 (1961).
9. H. Jenny, F. Bingman, and B. Padilla-Saravia, Soil Sci. 66, 173 (1948).
10. A. D. McLaren and K. Babcock IN T. Hayashi, "Subcellular Particles," Ronald Press, N. Y., 1959.
11. T. Hattori and C. Furusako, Nature 184, 1566 (1959).
12. H. Lees and J. H. Quastel, Biochemistry 40, 803 (1946).
13. A. D. McLaren, Science 141, 1141 (1963). The soil was first treated with 0.2% by weight of a polyelectrolyte in order to stabilize the crumb structure, air dried, crumbled and sieved in order to isolate the 2-5 mm fraction, and given a preliminary water wash. The washing treatment reduced the pH of the wet soil.
14. J. R. Freney, Nature 182, 1318 (1958).
15. J. R. Freney, Thesis, University of New England (1961).
16. S. M. Bromfield and V. B. D. Skerman, Soil Science 69, 337 (1950).
17. J. H. Quastel, E. J. Hewitt, and D. J. D. Nicholas, J. Agri. Sci. 38, 315 (1948).

18. J. I. Sperber, Aust. J. Agric. Res. 9, 782 (1958).
19. J. H. Quastel, "Nature," 171, 7 (1953).
20. A. D. McLaren, R. A. Luse, and J. J. Skujins, Soil Sci. Soc. Am. Proc. 26, 371 (1962).
21. J. J. Skujins, L. Braal, and A. D. McLaren, Enzymologia 25, 125 (1962).
22. A. D. Haig, Ph.D. Thesis, University of California, Davis, 1955.
23. A. D. McLaren, Archives Biochem. Biophys., 97, 1, April 1962, pp. 1-3.
24. D. M. Webley, D. J. Eastwood, and C. H. Giningham, J. Ecology 40, 169 (1952).
25. M. M. Kononova, Soil Organic Matter (Pergamon Press, New York, 1961).
26. M. H. Briggs and D. J. Spedding, Science Progress, April 1963, p. 217.
27. A. D. McLaren, IN Recent Progress in Microbiology VIII, University of Toronto Press, 1963.
28. G. H. Peterson, Soil Sci. 94, 71 (1962).
29. J. Bremner and K. Shaw, J. Ag. Sci. 44, 152 (1954).
30. J. Bremner, Biochem. J. 47, 538 (1950).
31. W. Van Driel, Studies on the conversion of amino acids in soil. North-Holland Publ. Co., Amsterdam, 1961.
32. E. Paul and E. L. Schmidt, S.S.S.A. Proc. 25, 359 (1961).
33. D. S. Jenkinson and G. Tinsley, Royal Soc., Dublin, Scientific Proc. 1, 141 (1960).
34. J. R. Freney, Aust. J. Ag. Res. 12, 424 (1961).
35. A. P. Adams, W. V. Bartholomew, and Francis E. Clark, S.S.S.A. Proc. 18, 40 (1954).
36. H. D. Putnam and E. L. Schmidt, Soil Sci. 87, 22 (1959).
37. C. A. Bower, Iowa Agr. Exp. Sta. Res. Bull. 363, August 1949.

38. D. J. Cosgrove, *Nature*, 194, 1265 (1962).
39. G. Anderson, *J. Soil Sci.* 12, 276 (1961).
40. F. J. Stevenson, *S.S.S.A. Proc.* 24, 472 (1960).
41. H. H. Johnson, *S.S.S.A. Proc.* 23, 293 (1959).
42. W. Flaig, *Proc. Natl. Acad. Sci. (India)* 24, 271 (1955).
43. C. B. Coulson, et al., *J. Soil Sci.* 11, 30 (1960).
44. A. C. Waldron and J. L. Mortensen, *S.S.S.A. Proc.* 25, 29 (1961).
45. J. R. Wright and M. Schnitzen, *Nature* 184, 1462 (1959).
46. J. R. Wright and M. Schnitzen, *Nature* 190, 703 (1961).
47. C. Steelink and G. Tollin, *B. B. Acta* 59, 25 (1962).
48. R. L. Whistler and K. W. Kirby, *J. Am. Chem. Soc.* 78, 1755 (1956).
49. D. L. Lynch et al., *J. Sci. Food and Agr.*, 1958, p. 56.
50. A. D. McLaren and D. Shugar, "Photochemistry of Proteins and Nucleic Acids," Pergamon Press, 1964.
51. H. F. Birch, *Plant and Soil* 10, 9 (1958).
52. D. Glaser, NAS Summer Work Shop, Stanford, 1964.
53. I. R. Kaplan and S. C. Rittenberg, *J. Gen. Microbiol.* 34, 195 (1964).

C. A Brief Survey of Enzymes Found
in Terrestrial Soil.

The presence of several kinds of enzyme activity in soil is well known, though only urease has been isolated and purified (1). While it has been shown that part of catalase-like activity in soil is of inorganic origin, there exists definite organic catalase-like enzymatic activity (6); in the case of phosphatase, all enzyme-like activity is of organic origin (3). The fact that enzyme activity has never been correlated with microbial numbers (2, 12, 15) leads us to believe that some enzymes may exist in soil in a free state, i.e., outside of microbial tissue.

In our search for extra terrestrial life our attention is turned to Mars (8), because its environment is most nearly like our own. However, with a thin atmosphere, high energy radiation must impinge on the surface. From soil sterilization studies (10, 11, 13) it has been shown that micro-organisms are more susceptible to high energy radiations than are different enzyme activities. Therefore, though low numbers of micro-organisms may be present on the Martian surface, the enzymes which had been excreted into the soil may still be present in measureable quantities.

From the foregoing discussion, it should be apparent that a positive test of enzyme activity on Mars could be interpreted as an indication of the presence of life. However, even at our advanced level of technology, we are unable to place large payloads on neighboring planets, which can operate for extended periods of time. Instead,

we must rely upon small instruments which give us the information desired in a short period. This constraint places a restriction upon the types of enzyme assay. In addition, small size limits the number of different enzymes we can look for and thereby forces us to choose from enzymes found in relatively high amounts in terrestrial soils and offering simple methods of analysis. It was in this context that our examination was undertaken.

Among the enzymes of earth, e.g. dehydrogenases, catalases, esterases, ureases, peroxidases, and phosphatases, little is known concerning the levels present in soil; by levels we mean moles of enzyme per unit weight of soil. As an answer to this question, the following enzyme activities were measured.

The enzymes examined, the reactions they catalyze, and the methods of determination of activities are summarized in Table I. A characterization of the soils examined and the results of microbial counts on these same soils are shown in Table II.

The results showing levels of enzyme per unit weight of soil are tabulated in Table III. [However, as Johnson and Temple (6) point out, in the case of catalase, the "peroxide-decomposing capacity" is not totally biological. Autoclaving soil reduced the decomposing capacity by $\frac{2}{3}$ while treatment with sodium azide lowered activity by $\frac{1}{3}$. Their figures would indicate that at least $\frac{1}{3}$ of their enzyme-like activity was non-biological.]

It has been suggested that enzymes are tied up with the organic fraction of soil and are perhaps present in amounts proportional to

the amount of organic matter (2, 6, 7, 12). The soils exhibit significant levels of substrate degradation, and all that needs to be done is to modify techniques to fit in a Martian probe (e.g. the Multivator). For example, a new fluorometric procedure for measuring dehydrogenase activity has been reported (4) which offers high sensitivity and does away with the soil-liquid separation step inherent in other colorimetric determinations. This is only very roughly borne out by a comparison of data in Tables II and III. Considering the activities of the enzymes tested in these soils, the most sensitive enzyme to test for is catalase. On the other hand, considering the ease of analytical procedure, dehydrogenase is perhaps the most useful. Urease offers a better possibility if C^{14} labelled urea is used as a substrate (see part A of this report.)

We now plan to study soils from deserts, soils stored for long periods in museums and soils from permafrost regions of the far North.

Table I
Enzymes, Reactions, and Methods of Analysis

| Enzyme | Reaction Catalyzed | Method of Determination | Reference |
|---------------|--|-----------------------------------|-----------|
| Dehydrogenase | Triphenyltetrazolium Chloride + $2H^+$ \longrightarrow Triphenyltetrazolium formazan + HCl | Colorimetric | (6) |
| Urease | Urea \longrightarrow $2 NH_3 + CO_2$ | Colorimetric | (3) |
| Esterase | Phenylacetate + H_2O \longrightarrow phenol + acetic acid | Colorimetric | (7) |
| Peroxidase | $H_2O_2 + 2[H] \xrightarrow[\text{acid}]{\text{ascorbic}} 2 H_2O$ | Titrate residual H_2O_2 | (6) |
| Catalase | $2 H_2O_2 \longrightarrow 2 H_2O + O_2$ | Titrate residual H_2O_2 | (2) |
| Phosphatase | Na- β -naphthyl phosphate \longrightarrow β -naphthol + PO_4^{3-} | β -naphthol fluorescence | (8) |

Table II
Soil Characteristics

| Soil Type | Characteristics | Microorganisms/ gram soil | pH | Percent organic matter |
|------------|---|------------------------------|-----|------------------------------|
| Aiken | Heavy textured clay loam; stored. | 2×10^5 | 5.8 | 13.5% |
| Columbia | Granular medium textured porous; subsoil; stored. | 1.0×10^5 | 6.8 | 7.5% |
| Dublin | Loam; high moisture retaining capacity; stored. | 6×10^5 | 5.6 | 14.5% |
| Yolo | Structureless, loose sandy loam; stored. | 1.0×10^5 | 7.0 | 9.3% |
| Greenhouse | Loam (fresh). | 4.0×10^7 | 6.7 | 13.3% |
| Creek | Silty. | 3.0×10^7 | 7.1 | 6.5% |

Table III
Ratio Grams Enzyme/Gram Soil For Soils Tested*

| Dehydrogenase | | Urease | |
|---------------|-----------------------|----------|-----------------------|
| Aiken | $.93 \times 10^{-7}$ | Aiken | 3.1×10^{-9} |
| Columbia | $.77 \times 10^{-7}$ | Columbia | 3.1×10^{-9} |
| Dublin | 1.46×10^{-7} | Dublin | 3.5×10^{-9} |
| Yolo | 1.70×10^{-7} | Yolo | 3.25×10^{-9} |
| Greenhouse | 3.47×10^{-7} | | |
| Creek | 4.60×10^{-7} | | |

| Peroxidase | | Catalase | |
|------------|-----------------------|----------|----------------------|
| Aiken | 3.0×10^{-7} | Aiken | $.6 \times 10^{-7}$ |
| Columbia | 4.75×10^{-7} | Columbia | 3.4×10^{-7} |
| Dublin | 5.75×10^{-7} | Dublin | 5.2×10^{-7} |
| Yolo | 5.25×10^{-7} | Yolo | 5.6×10^{-7} |

| Phosphatase | | | |
|-------------|-----------------------|--|--|
| Aiken | $.25 \times 10^{-7}$ | | |
| Columbia | $.22 \times 10^{-7}$ | | |
| Dublin | 1.62×10^{-7} | | |
| Yolo | $.34 \times 10^{-7}$ | | |

* Weight of enzyme is calculated from known activities of purified, often crystallized enzymes reported in the 1963 catalog of the Worthington Biochemical Company, N.J.

References

1. Briggs, Spelding. "Soil Enzymes". Science Progress 51, 217 (1963).
2. Casida, L.E., Klein, D.A., Santoro, T. "Soil Dehydrogenase Activity". Soil Sci. 98, 371 (1964).
3. Eno, C.F., Popenoe, H. "Gamma Radiation Compared with Steam and Methyl Bromide as a Soil Sterilizing Agent". Soil Sci. Soc. Am. Proc. 28, 533 (1964).
4. Guilbault, G.C., Kramer, D.N. "New Direct Fluorometric Method for Measuring Dehydrogenase Activity". Analyt. Chem. 36, 2497 (1964).
5. Haig, A.D. Ph.D. Thesis, Univ. of Calif. Davis, "Some Characteristics of Esterase and Urease-Like Activity in Soil". (1955).
6. Johnson, J.L., Temple, K.L. "Some Variables Affecting the Measurement of 'Catalase Activity' in Soil". Soil Sci. Soc. Am. Proc. 28, 207 (1964).
7. Kozlov, K. "Enzymatic Activity of the Rhizosphere and Soils in the East Siberia Area". Folia Mikro 9, 145 (1963).
8. Moore, P. "Guide to Mars". Muller, Great Britain (1956).
9. Moss, D.W., Campbell, D.M., Anagnostoukarakas, E., King, F. "Characterization of tissue alkaline phosphatase and partial purification by starch gel electrophoresis". Biochem. J. 81, 441 (1961).
10. McLaren, A.D., Retshetko, L., Huber, W. "Sterilization of Soil by Irradiation with an Electron Beam and Some Observations on Soil Enzyme Activity". Soil Sci. 83, 497 (1957).
11. McLaren, A.D., Luse, R.A., Skujins, J.J. "Sterilization of Soil by Irradiation and Some Further Observations on Soil Enzyme Activity". Soil Sci. Soc. Am. Proc. 26, 371 (1962).
12. Nowak, W. "Vergleichsstudien zwischen dem Mikroorganismengehalt des Bodens und seiner Saccharaseaktivität". Plant and Soil XX, 302 (1964).
13. Skujins, J.J., Braal, L., McLaren, A.D. "Characterization of Phosphatase in a Terrestrial Soil Sterilized with an Electron Beam". Enzymologia XXV, 125 (1962).

14. Snell, Snell. "Colorimetric Methods of Analysis".
D. van Nostrand and Co., New York (1937).
15. Stevenson, I.L. "Dehydrogenase Activity in Soils". Canad.
J. Micro. 5, 229 (1959).
16. Worthington Biochemical Corporation, Catalog, 1963.

D. Urease Activity in Media of Low Water Content.

This report describes the exploratory investigations regarding the action of urease on urea in saturated solutions. Extensive literature search has shown so far that there exists virtually no information concerning urease activity under these conditions.

The work described herein is mainly concerned with an examination of experimental approaches to the study of the problem and gives initial results regarding urease activity in concentrated solutions. The study is particularly concerned with a sensitive assay of urease activity in preparation for urease measurements in soil. Pertinent information found in literature is included and reviewed.

PERTINENT INFORMATION ON UREA.

In order to properly approach the study of urease action on urea in concentrated solutions, certain aspects of the urea chemistry should be considered.

Chemical constitution. Urea generally is considered as the diamide of carbonic acid, or carbamide, $\text{NH}_2 - \text{CO} - \text{NH}_2$. Considerable evidence has been gathered by Werner (1) to show that urea exists in a cyclic form $\text{HN} = \text{C} \begin{array}{c} \text{NH}_3 \\ | \\ \text{O} \end{array}$; he visualizes a transient existence of an active tautomeric enol form, $\text{HN} = \text{C} \begin{array}{c} \text{NH}_2 \\ | \\ \text{OH} \end{array}$, which has been stabilized in certain isoureas. These views are supported by the fact that urea often acts as a monobasic substance. However, in many organic reactions it is difficult to justify the cyclic structure and the carbamide structure of urea prevails.

Stability (2). Decomposition of urea in cold aqueous solutions is negligible, but upon boiling it is converted to ammonium cyanate. The cyanate is then hydrolyzed to ammonium carbonate. Rapid hydrolysis is observable already at $50^{\circ} - 60^{\circ}\text{C}$. In cold dilute acids and alkalies urea is not decomposed, but on heating complete decomposition to NH_3 and CO_2 is effected. The decomposition is more rapid in acid solutions.

In ammonia containing solutions, urea reacts with alkali metals and magnesium with the evolution of hydrogen (Note: rapid gas development was observed when concentrated urea solution was placed on an aluminum planchet).

Crystalline addition compounds (2). Urea forms addition compounds with acids, acting as a monobasic compound. Familiar forms are those with nitric, sulfuric, and oxalic acids; acetic acid forms large deliquescent crystals, $\text{CO}(\text{NH}_2)_2 \cdot \text{CH}_3\text{COOH} \cdot 2\text{H}_2\text{O}$; only one urea phosphate derivative exists. Crystalline addition compounds are formed with a large number of inorganic salts and organic compounds, including salts of heavy metals. Similarly, urea replaces the water of crystallization in many compounds.

It should be noted that in an alkaline solution, in presence of ammonia, the compound $\text{NH}_3 \cdot \text{CO}(\text{NH}_2)_2$ possibly exists, hampering methods for the analysis of ammonia present (3), (25).

Solubility. The solubility of urea in water, as determined by Shnidman and Sunier (4) is shown in Fig. 1. At room temperature there are 3 molecules of water per molecule of urea in a saturated solution. Urea is deliquescent. The hygroscopicity of urea has been examined by Oka (5).

Vapor pressure. The vapor pressure of saturated aqueous solutions at the room temperature range was determined by Edgar and Swan (6), Fig. 2; for comparison, vapor pressure of pure water is also shown (7).

MATERIALS AND DEVELOPMENT OF METHODS.

Urea (Merck, reagent grade). After obtaining repeatedly inconsistent results with the reagent grade urea and recrystallized reagent grade urea, it was apparent that excessive amounts of ammonium ion and possibly other impurities were present in the saturated urea solutions.

A purification method, utilizing ion exchange resins was adapted, as first suggested by Benesch et al., (8):

An ion exchange column was filled with AG 501-X8 (BioRad, analytical grade) mixed bed resin, and concentrated urea solution (approx. 140g/100 ml glass dist. water) was eluted at elevated temperature, 40° - 50°C. Eluate was collected in a vessel immersed in an ice bath and urea was crystallized in the refrigerator at 3°C overnight. The urea crystals were filtered in the cold under suction, washed with cold acetone and dried at room temperature. Subsequent tests indicated that the ion exchange column treated urea was essentially free of NH_4^+ and other ions. A second yield of crystals was obtained by further cooling of the mother liquor to - 30°C; but this second fraction showed an increased amount of impurities and was not used.

Urease (NBC, 3xNF). For the presently described exploratory experiments, a non-crystalline urease preparation was used in water or buffer suspensions, 0.1 to 1.0 mg/ml. In order to improve the experimental conditions it was attempted to dissolve urease in aqueous solution and to separate the insoluble protein portion by centrifugation. However, the results were negative, as after the

centrifugation most of the activity was associated with the insoluble fraction and only a minimal amount of the activity was found in the supernatant.

The molecular weight of urease is 483,000 and the turnover number 460,000.

General method for urea-urease incubation. The determination of the urease activity was based on the following method:

To an excess amount of concentrated urea solution in water or in a desired buffer add urease suspension and incubate at known temperature for a predetermined length of time. Analyze for ammonia by eluting an aliquot through a cation exchange resin, thus separating NH_4^+ from unreacted urea; subsequently eluting NH_4^+ from the resin and analyzing by nesslerization.

An alternative, indophenol blue method was developed for ammonia analysis directly in the urea containing solution, but this method was valid only if no traces of reducing agents (e.g. cysteine) were present.

Reagents:

Buffer: Na-acetate, 0.05 M, pH 5.5, containing 0.025% cysteine, obtained by titrating 0.05 M Na-acetate with glacial acetic acid.

Na-citrate was prepared similarly.

Urea solution: 50% W/V, in Na-acetate buffer, dissolved at temperatures below 25°C to avoid decomposition; diluted with buffer for lower concentrations.

Urease: 0.1 to 1.0 mg/ml, suspended in distilled water and heated to 60°C for 5 minutes before use.

Normally 6 ml of urea solution in buffer was incubated with 1 ml of urease suspension for 5 minutes at room temperature.

For exploratory experiments the amounts and volumes of above reagents, as well as pH and incubation periods were varied to establish conditions for maximum urease activity.

After a predetermined incubation time, an equal volume of 16% trichloroacetic acid or, preferably, 0.01 N HCl was added to inactivate the urease. For further analysis the urea-urease solution was titrated with NaOH to pH 7.0 and made up to a known volume, usually 10 ml.

Analysis of ammonia by the ion exchange method as initially described by Kistiakowsky et al. (9).

A Dowex 50-X8 cation exchange resin, - 400 mesh, H^+ form, was changed to Na^+ form by washing it with 0.1 N NaOH, and subsequent washing with excess 1% NaCl. Pyrex semi-micro funnel No. 26290-3C was used as the ion exchange column. The column was filled with Na^+ form of Dowex-50 to 10 mm height, washed with glass distilled water until the eluate was neutral; 1 ml of neutralized urea-urease solution was added on the column, after 5 minutes reaction time the column was washed with 10 ml glass distilled water, and the adsorbed NH_4^+ was subsequently eluted with 10 ml 0.02 N NaOH. To the 10 ml of the NaOH eluate, 1 ml gum acacia solution was added and nesslerized with 1 ml Nessler's solution. The absorbance was read at 420 m μ by using the "Spectronic 20" spectro-photometer. The recovery varies between 95% and 105%.

Analysis of ammonia by the indophenol blue method.

This method has been described by Bolleter et al (10), and independently at the same time applied to the ammonia analysis in soil extracts by Hoffmann and Teicher (11). The method was adapted as follows:

Reagents:

Phenol reagent: dissolve 62.5 g. phenol in about 10 ml ethanol to which 2 ml methanol has been added; add 18.5 ml acetone and make volume to 100 ml with ethanol. Store in refrigerator.

Alkaline phenol reagent: mix 20 ml of phenol reagent with 20 ml of 6.75 N NaOH before use; make volume to 100 ml with water.

Sodium hypochlorite solution: Dilute available NaOCl solution to 0.9% active (i.e. available) Cl content (if it is necessary to determine the available Cl content, the arsenious oxide titration method should be used as described in "Official Methods of Analysis, A.O.A.C.").

Method:

In a 150 x 18 mm photometer tube introduce in the following order:

- 1.0 ml sample,
- 10.0 ml water,
- 1.0 ml alkaline phenol reagent, mix thoroughly,
- 1.0 ml 0.9% Na-hypochlorite, mix thoroughly.

After 30 minutes read at 630 mμ in "Spectronic 20." A straight line relationship of ammonia concentration vs. absorbance is obtained up to 0.05 mg NH₃-N/ml with an absorbance reading of about 0.550.

Determination of urease activity by the analysis of CO₃⁼.

Preliminary data indicate that it might be possible to determine the released CO₃⁼ by precipitating it with Ba⁺⁺; however, presently this analytical method has not been pursued further.

Standardization of the available Geiger-Mueller detectors for C¹⁴ counting.

The C^{14} counting characteristics were determined for the Nuclear-Chicago Corporation's G-M gas flow tube, Model D47, and the Forro Scientific Company's G-M gas flow tube, Model FS; both were used with the Nuclear-Chicago Corporation decade scaler, Model 186A.

The detector tubes were standardized against a Baird-Atomic, Inc., C^{14} -standard reference disc. No. 9-3222, active window diameter $3/4"$, window cover 0.9 mg/cm^2 , activity $0.189 \pm 5\% \mu\text{c}$; equivalent to 421800 disintegrations/minute.

Gas: Q-gas: 98.7% helium, 1.3% butane.

Sensitivity setting on scaler: 250 mV.

Nuclear-Chicago gas flow detector tube:

window: "Micromil" 0.15 mg/cm^2 ,

effective counting area: 1134 mm^2 (38 mm diameter),

operational voltage, as determined from the voltage plateau: 1250 V.

Counts against the B-A standard: 30.5 mm distant: 18,800 cpm,

40.5 mm distant: 9,750 cpm,

35.0 mm distant (interpolated):
14,000 cpm,

coincidence limit = 45,000 cpm.

Forro FS gas flow detector tube:

window: mylar, 0.8 mg/cm^2

effective counting area: 530 mm^2 ; counting area with the plastic

support, as used for standardization: 490 mm^2 .

operational voltage, as determined from the voltage plateau: 1250 V.

Counts against the B-A standard: 18.0 mm distant: 21,233 cpm,

35.0 mm distant: 5,726 cpm.

Actual efficiency of the Forro FS detector as compared with the Nuclear-Chicago detector:

$$\frac{\frac{530 \text{ mm}^2}{490 \text{ mm}^2} \times 5726 \text{ cpm}}{14000 \text{ cpm}} = 0.44$$

Relative efficiency per sensitive counting area of Forro FS detector as compared with the Nuclear-Chicago detector:

$$\frac{\frac{1134 \text{ mm}^2}{490 \text{ mm}^2} \times 5726 \text{ cpm}}{14000 \text{ cpm}} = 0.95$$

In comparing the efficiencies it should be noted that the cover for the B-A standard, 0.9 mg/cm^2 , is thicker than either of the G-M tube windows.

Development of the self-contained C^{14}O_2 counting chamber.

A self-contained radioactive gas counting chamber has been designed and made as shown in Fig. 3. The chamber is designed for the use with the Forro FS G-M tube or with a solid state radiation detector. It is made of thick vacuum-flask Pyrex glass and an outlet is provided for an atmospheric pressure and/or moisture control. The inner volume of the chamber is 360 cm^3 . A thin stainless steel plate is used for shielding the detector from direct radiation from the radioactive sample. Presently no additional stirring apparatus has been provided to facilitate the gas diffusion within the chamber. A complete diffusion of gas from the sample is achieved in 10 to 15 minutes in the chamber. Characteristics for counting of C^{14}O_2 in the chamber are shown in Fig. 4; the standardization curve was obtained by reacting $12.5 \text{ } \mu\text{C}$ of $\text{NaHC}^{14}\text{O}_3$ with excess H_2SO_4 . $12.5 \text{ } \mu\text{C}$ C^{14}O_2 in the

chamber atmosphere ($0.035 \mu\text{c}/\text{cm}^3$) gave 34200 cpm after equilibration, or 2738 cpm/ μc of C^{14}O_2 in the atmosphere. The count was equivalent to 980 cpm/ $0.001 \mu\text{c}/\text{cm}^3$.

The background atmospheric C^{14}O_2 count in the chamber is dependent on the pH of the carbonate solution, i.e., the equilibrium between CO_2 in the air and $\text{CO}_3^{=}$ in the solution. In a test case 12.5 μc of $\text{NaHC}^{14}\text{O}_3$ were dissolved in 50 μl 0.01 N NaOH. The self decomposition of the carbonate under these conditions was a linear function, reaching 1500 cpm in 14 minutes.

Tests for C^{14}O_2 released from saturated urea solutions by urease were performed as follows: 10.0 mg of purified urea, enriched with C^{14} -labeled urea to contain 1 $\mu\text{c}/\text{mg}$ were weighed in a stainless steel planchet and placed in the chamber and shielded; 10 μl of 1 mg/ml urea solution in appropriate buffer were added onto the dry urea by injecting it with a syringe. The appearance of C^{14}O_2 in the chamber atmosphere was monitored with the Ferro FS detector tube.

The development of the solid state radiation detector.

The solid state radiation detector is being developed with the assistance from the LRL Nuclear Instrumentation Chemistry Group (Mr. Fred Goulding's group) and the Soils and Plant Nutrition departmental shop (Mr. C. Brown).

The investigation for the applicability of a solid state radiation detector for the current project was deemed desirable mainly due to the following two reasons:

1. The solid state detecting unit requires considerably less space than the G-M tube; it is more versatile in geometrical positioning with respect to the monitored sample, and does not need quench gas attachments.

2. Its efficiency is increased in low pressure (vacuum) conditions, where the G-M tubes cannot be used because of pressure differences.

It should be recognized that the solid state detectors are considerably less sensitive than the G-M tubes in terms of count rate. Attempt has been made to incorporate a solid state detector in the Gulliver project, but the idea was discarded because of the low sensitivity (12). However, it is understood that a lithium-silicone drifted junction type detector has been tried in the Gulliver project, whereas presently phosphorus-silicone diffused junction type detectors are being tried.

A phosphorus-silicone diffused junction type detector ("an inferior quality discard") was obtained from the LRL* and a detector unit, adaptable to the self-contained $C^{14}O_2$ counting chamber was made. The $C^{14}O_2$ counting efficiency with the solid state detector in the self-contained chamber was tested with the LRL group's scaler and channel analyzer equipment.

From a rough test run at the LRL the following values were obtained: total $C^{14}O_2$ count in 15 minutes from a $50\mu c NaHC^{14}O_3 - H_2SO_4$ source: 11,156; total background at the same time: 3450.

In order to adapt the solid state detector to the available Nuclear-Chicago scaler, an appropriate preamplifier and a power supply are being made.

* Lawrence Radiation Laboratory

RESULTS

Hydrolysis of urea as determined by the ammonia analysis.

In the course of the examination of the problem of urease action on urea in concentrated and saturated urea solutions, many various conditions were tested with respect to urea and urease concentrations, types of buffers (Na and K phosphates, acetates, and oxalates) at several pH values and concentrations, presence or absence of protecting agents (cysteine, glutathione), and incubation times. The incubation temperature was, however, kept at "room temperature," about 22°C, to avoid non-enzymatic hydrolysis of urea. Fresh urea solutions were used, however, for all urease-urea experiments, as it was shown that analytically detectable hydrolysis of urea occurred upon standing at room temperature for 24 hours. Hydrolysis of urea was negligible when the urea solutions were kept at 3°C. Also, the initial experiments were done with commercial "reagent grade" urea; later, ion-exchange-column purified urea was used.

Analytical methods included nesslerization after the separation of ammonia from the urease-urea solution by Conway method or by the ion exchange column; also, indophenol-blue method was used.

Effects of the various conditions on the urea hydrolysis by urease are discussed below.

All results obtained under the various conditions, most of which were exploratory and semi-quantitative, however, may be presently combined to show a "composite activity curve" of the urease action on urea (Fig. 5). It has been shown that there is a definite positive hydrolytic activity of urease on urea in a saturated solution, as yet however, quantitatively unspecified.

Hydrolysis of urea as determined by $C^{14}O_2$ analysis.

Saturated solutions of C^{14} -urea were decomposed by urease at room temperature in the self-contained radioactive gas counting chamber. The development of $C^{14}O_2$ was monitored and the rate of urea hydrolysis appeared to be a logarithmic function with respect to time, $t; t = \frac{ka}{e}$ (Fig. 6). In 5.25 hours 8.9% of the available C^{14} (from 10.0 mg urea, containing $10 \mu C^{14}$, 50% by weight in water solution) appeared as $C^{14}O_2$ in the chamber atmosphere, represented by a count of 2343 cpm. In a control experiment the non-enzymatic hydrolysis of urea appeared to be a linear function and produced 292 cpm in 5.25 hours.

The atmospheric moisture content was not controlled in these experiments, and some crystallization of urea or the hydrolytic products was observed on the rims of planchets at the time of the completion of the tests.

Ammonium carbamate as the substance for urease.

Several experiments were performed to test if ammonium carbamate were a substrate for urease, but all gave negative results.

DISCUSSION

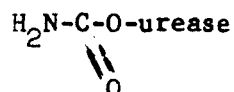
Chemistry of urea hydrolysis by urease.

A complete hydrolysis of urea by urease requires 2 moles of water per 1 mole of urea: $NH_2CONH_2 + 2H_2O \longrightarrow (NH_4)_2CO_3$. Apparently it is a 2-step enzymatic hydrolysis reaction, and several investigators have made studies of possible intermediates. This question, however, has not been completely resolved as yet.

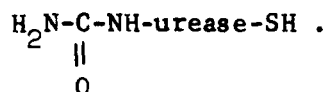
Sumner et al., (13) have shown that cyanate is not an intermediate, but ammonium carbamate appears when urea is acted on by urease in

alkaline solution; in a neutral phosphate buffer or in slightly acidic solution carbamate does not appear. It should be noted that carbamate hydrolyzes spontaneously at pH values below 7.

An existence of an intermediate substrate-enzyme complex



was first suggested by Brandt (14). Bersin (15) has suggested a somewhat different form for the intermediate complex:



In order to avoid the interference by carbamate as the reaction product, the experiments described herein were performed in buffers at pH values below pH 6.0.

Influence and selection of various factors for urease activity.

No typical activators are known for urease. However, several types of compounds have been used as "protectors" for urease, especially the mild reducing agents, as cysteine, glutathione, NaHSO_3 , and H_2S ; also gum acacia has been used as a stabilizing agent. It is evident that the active center of urease is associated with the -SH groups on the urease molecule, nevertheless, there exists evidence that the urease activity is independent of the redox potential in the medium (16).

The activity of urease is strikingly dependent on the composition of the buffer employed. An extensive study of the influence of buffers was done by Howell and Sumner, Fig. 7 (17).

Aside from the highly purified, water-soluble crystalline urease, this enzyme is regularly available as "insoluble" urease in powdered form, containing various nonspecified proteins. Unsuccessful attempts

were made to solubilize the active urease fraction in water or in several buffers; after intensive stirring and centrifugation virtually all of the urease activity was found in the sediment. The preparation of insoluble but active urease was first described by Sumner and Graham (18) and the denaturation of urease without its inactivation was investigated by Sumner (19). Dispersed insoluble urease was used throughout for the experiments described herein. The specific gravity of the concentrated urea solutions was sufficiently high to maintain urease in well dispersed state without sedimentation. The use of insoluble enzyme with a soluble substrate presents an interesting facet of the current investigation.

The activity of urease suspensions and solutions may be increased by short time heating at elevated temperatures immediately before use. For the current investigation, the insoluble urease suspensions were heated for 5 minutes at 60°C. This phenomenon was investigated by Hofstee (20) who suggested that a reversible association-dissociation, dependent on temperature might occur between the urease molecules in solution. The heterogenous nature of urease in solution was shown also by McLaren et al., (21) with the ultracentrifuge sedimentation methods. Creeth and Nichol (22) reinvestigated the problem, and concluded that dimers and trimers tend to be formed in urease solutions, but that the system is not one in rapid equilibrium; the polymers are probably formed by intermolecular disulphide cross linkages.

Urease is strongly inhibited by a large number of heavy metal ions. The stabilization of urease activity in solution by H_2S and similar reagents might be at least in part interpreted as a result of

precipitation of heavy metal ions by these reagents. It has been shown that in phosphate buffers Na^+ inhibits the urease activity more than K^+ at the same concentration (23). This problem, and also the inhibition by other common ions, has been reinvestigated by Kistiakowsky et al., (9), who indicated that inhibitory mechanisms, other than the commonly known salt effect, may be present.

The apparent inhibition of urease by increased concentrations of urea was clearly demonstrated by our experiments (Fig. 5). The inhibition of urease by the substrate has been investigated by Hoare and Laidler (24) from the point of view of enzyme kinetics and activation energy. It has been suggested by Deasy (25) that a compound $\text{NH}_3 \cdot \text{CO}(\text{NH}_2)_2$ might be present at higher urea concentrations and that it might act as an inhibitor. The kinetics of urea hydrolysis by urease has been re-examined by Kistiakowsky and Rosenberg (26).

It might become of interest to examine the kinetics and inhibition of urease in the light of more recently accumulated information regarding substrate-induced enzyme inactivation (e.g., ref. 27).

Information contained in a forthcoming book, Proceedings of the Conference on Forms of Water in Biological Systems (28) might also prove of value for the interpretation and evaluation of our results.

There is some evidence that deuterated water exists on Mars (29), thus, it should be of interest to examine the activity of urease in heavy water. Some initial work on this aspect has been done by Jacobsohn and Azevedo (30).

References

1. Werner. The Chemistry of Urea, Longmans, Green and Co., London, 1923.
2. Berliner, J.F.T. Ind. Eng. Chem. 28, 517 (1936).
3. Jhnecke, E., and Rahlfs, E.Z. Elektrochem. 36, 645 (1930).
4. Shnidman, L., and Sunier, A.A. J. Phys. Chem. 36, 1232 (1932).
5. Oka, S. J. Soc. Chem. Ind. (Japan), 33, 227 (1930).
6. Edgar, G., and Swan, W.O. J. Am. Chem. Soc. 44, 570 (1922).
7. Hodgman, M.S., Weast, R.C., and Selby, S.M., eds., Handbook of Chemistry and Physics. 37th edition, Chemical Rubber Publishing Company, Cleveland, Ohio, 1955, pp. 2142-2143.
8. Benesch, R.E., Lardy, H.A. and Benesch, R. J. Biol. Chem. 216, 663 (1955).
9. Kistiakowsky, G.B., Mangelsdorf, P.C. Jr., Rosenberg, A.J., and Shaw, H.R. J. Am. Chem. Soc. 74, 5015 (1952).
10. Bolleter, W.T., Bushman, C.J., and Tidwell, P.W. Anal. Chem. 33, 592 (1961).
11. Hoffmann, G., and Teicher, K. Z. Pflanzenern. Dung. Bodenk. 95, 55 (1961).
12. Horowitz, N. Personal communication, August 8, 1964.
13. Sumner, J.B., Hand, D.B., and Holloway, R.G. J. Biol. Chem. 91, 333 (1931).
14. Brandt, Biochem. Z. 291, 99 (1937).
15. Bersin, Kurzes Lehrbuch der Enzymologie, Akademische Verlagsanstalt, Leipzig, 1954, cited in Ammon, R., and Dirscherl, W., Fermente, Hormone, Vitamine, vol. I, Georg. Thieme Verlag, Stuttgart, 1959, p. 303.
16. Fischgold, Biochem. J. 28, 406 (1934).

17. Howell, S.F., and Sumner, J.B. J. Biol. Chem. 104, 619 (1934).
18. Sumner, J.B., and Graham, V.A. Proc. Soc. Exp. Biol. Med. 22, 504 (1925).
19. Sumner, J.B. Science 108, 410 (1948).
20. Hofstee, B.H.J. J. Gen. Physiol. 32, 339 (1949).
21. McLaren, A.D., Sheppard, E., and Wagman, J. Nature 162, 370 (1948).
22. Creeth, J.M., and Nichol, L.W. Biochem. J. 77, 230 (1960).
23. Fasman, G.D., and Nieman, C. J. Am. Chem. Soc. 73, 1646 (1951).
24. Hoare, J.P., Laidler, K.J. J. Am. Chem. Soc. 72, 2487 (1950).
25. Deasy, C.L. J. Am. Chem. Soc. 69, 294 (1947).
26. Kistiakowsky, G.B., and Rosenberg, A.J. J. Am. Chem. Soc. 74, 5020 (1952).
27. Grisolia, S. Physiol. Rev. 44, 657 (1964).
28. Cited in Science, 147, 179 (1965).
29. Shirk, J.S., Haseltine, W.A., and Pimentel, G.C. Science 147, 48 (1965).
30. Jacobsohn, K.P. and Azevedo, M.D. Revista Fac. Ciencias, Univ. Lisboa, 2^a Ser. B 1, 65 (1953).

FIG. 1.
Solubility of urea
in water
(Shnidman and Sunier, 1932)

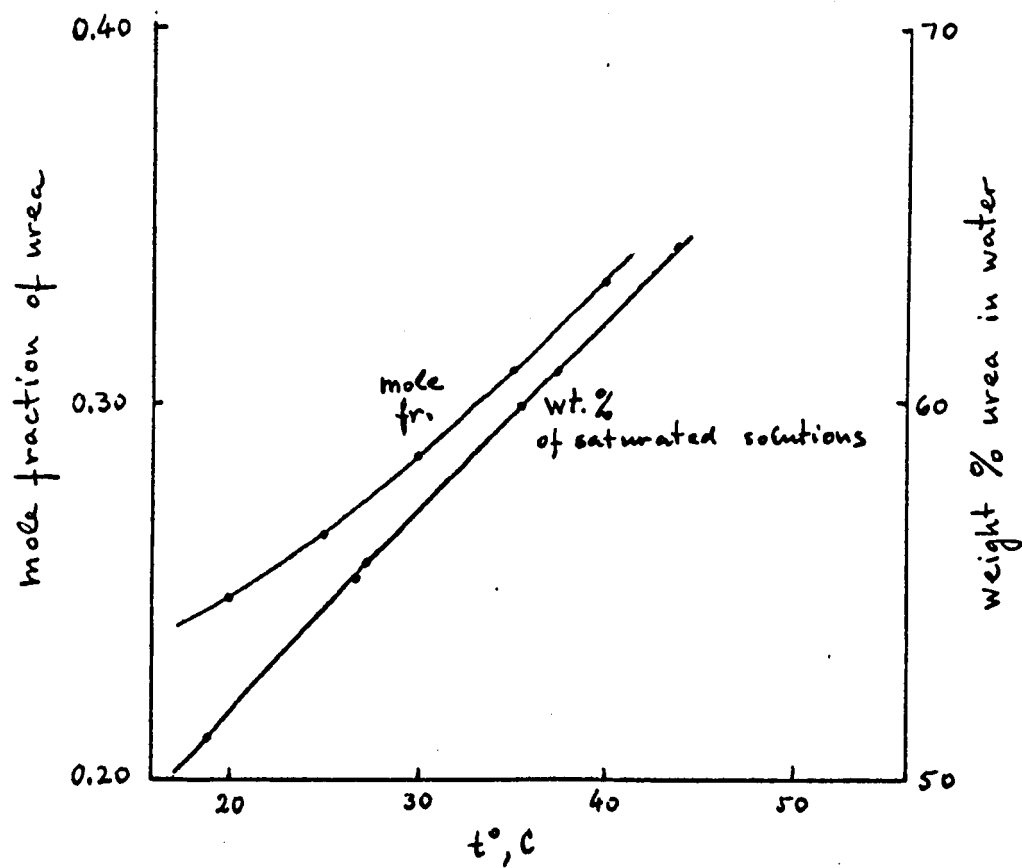


FIG. 2.

Vapor pressure of saturated aqueous urea
(Edgar and Swan, 1922)

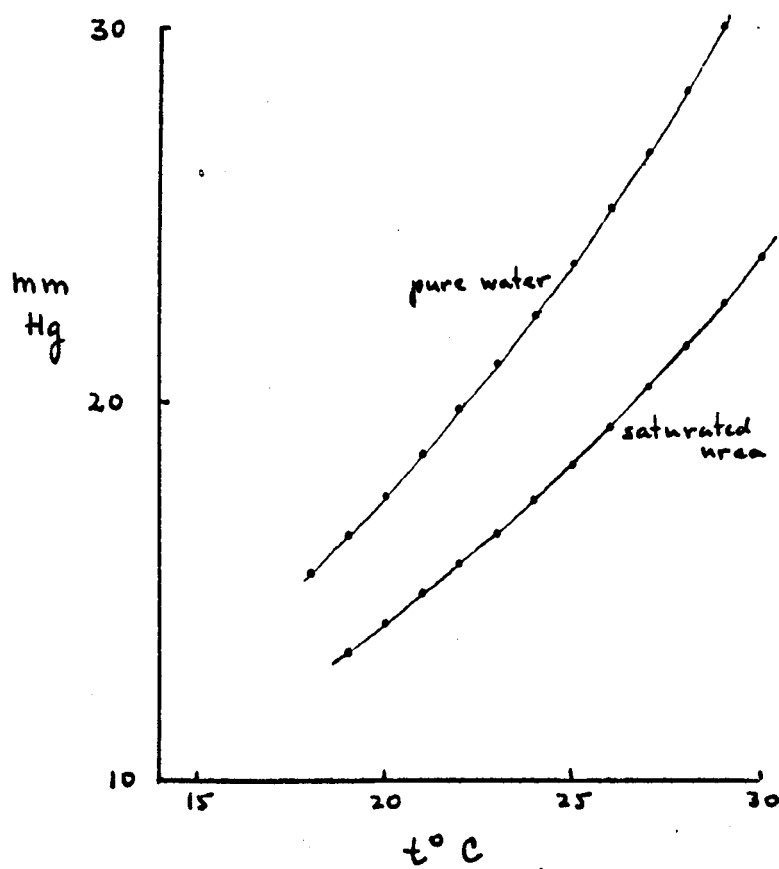


FIG. 3.

The self-contained radioactive gas counting chamber.

Schematic cross-section

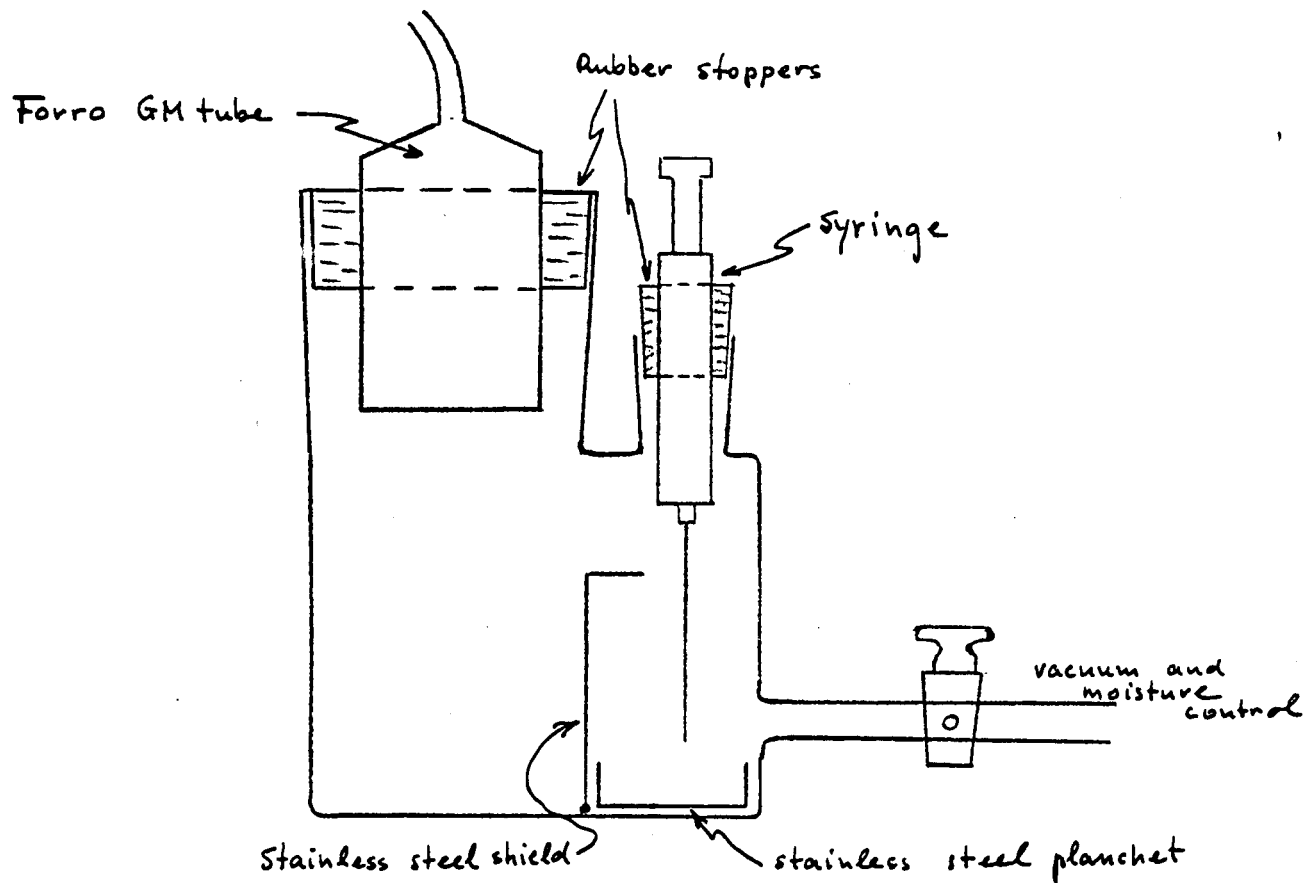
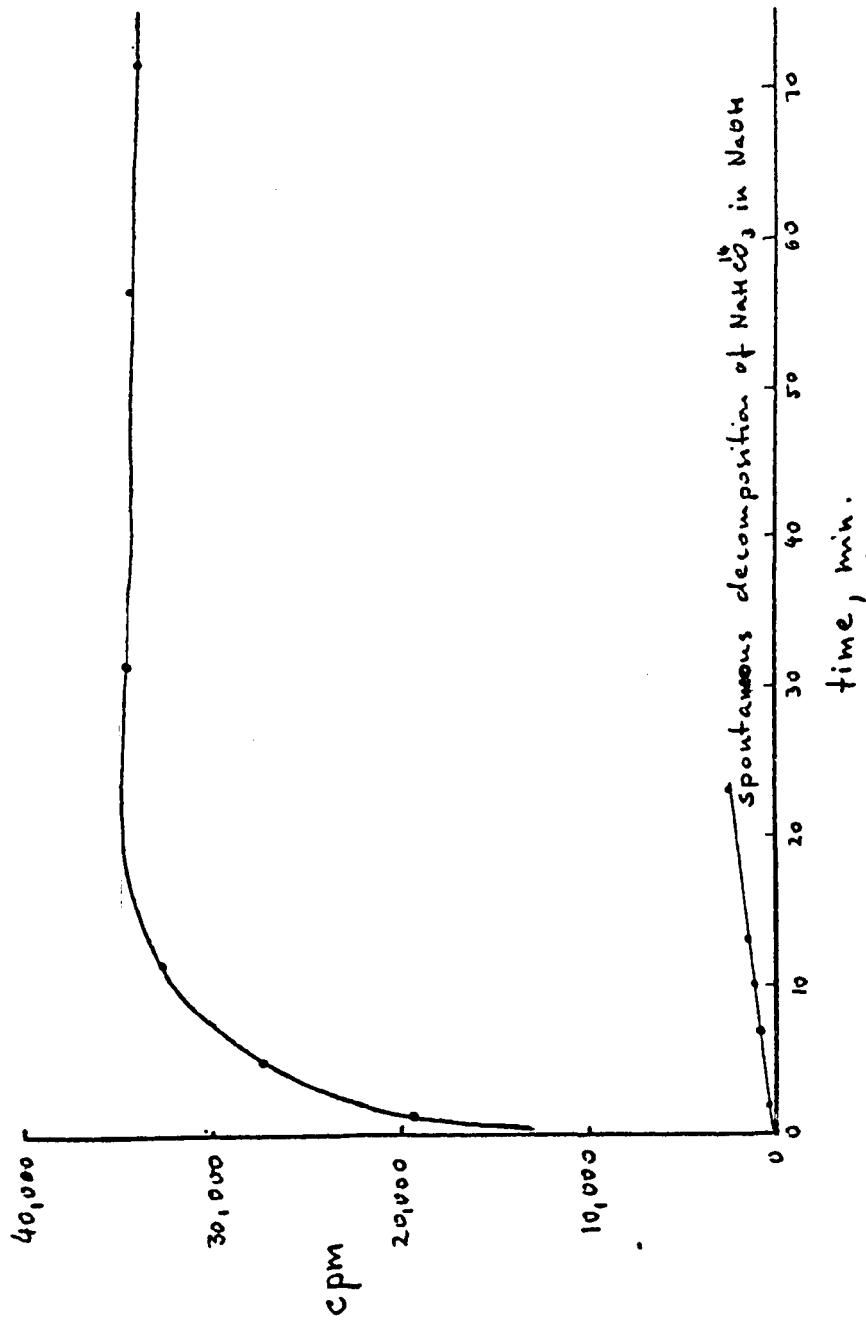
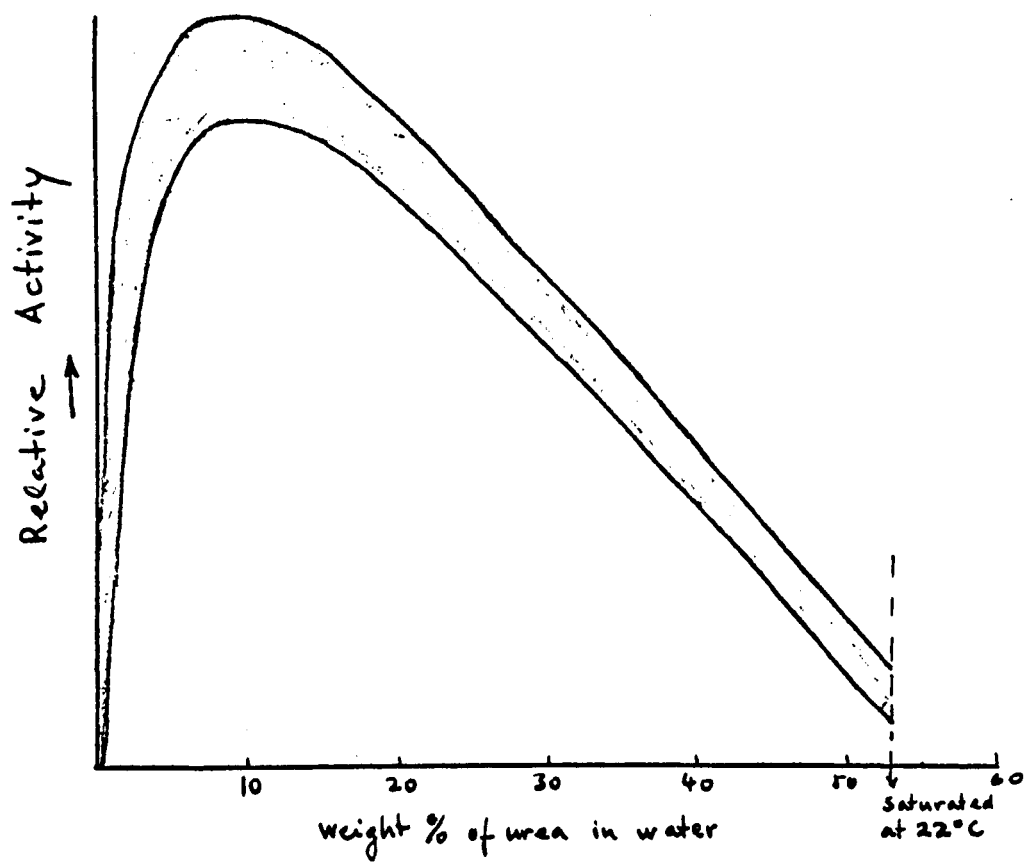


FIG. 4.



C^{14}O_2 yield in the chamber
 from a $12.5\mu\text{e NaH}^{14}\text{CO}_3\text{-NaOH}$
 source treated with H_2SO_4

Fig. 5.



A composite representation of urease activity on urea at various conditions; room $t^{\circ} = 22^{\circ}\text{C}$.

FIG. 6

Development of $C^{14}O_2$ by the action of urease on C^{14} -urea
in a saturated solution.

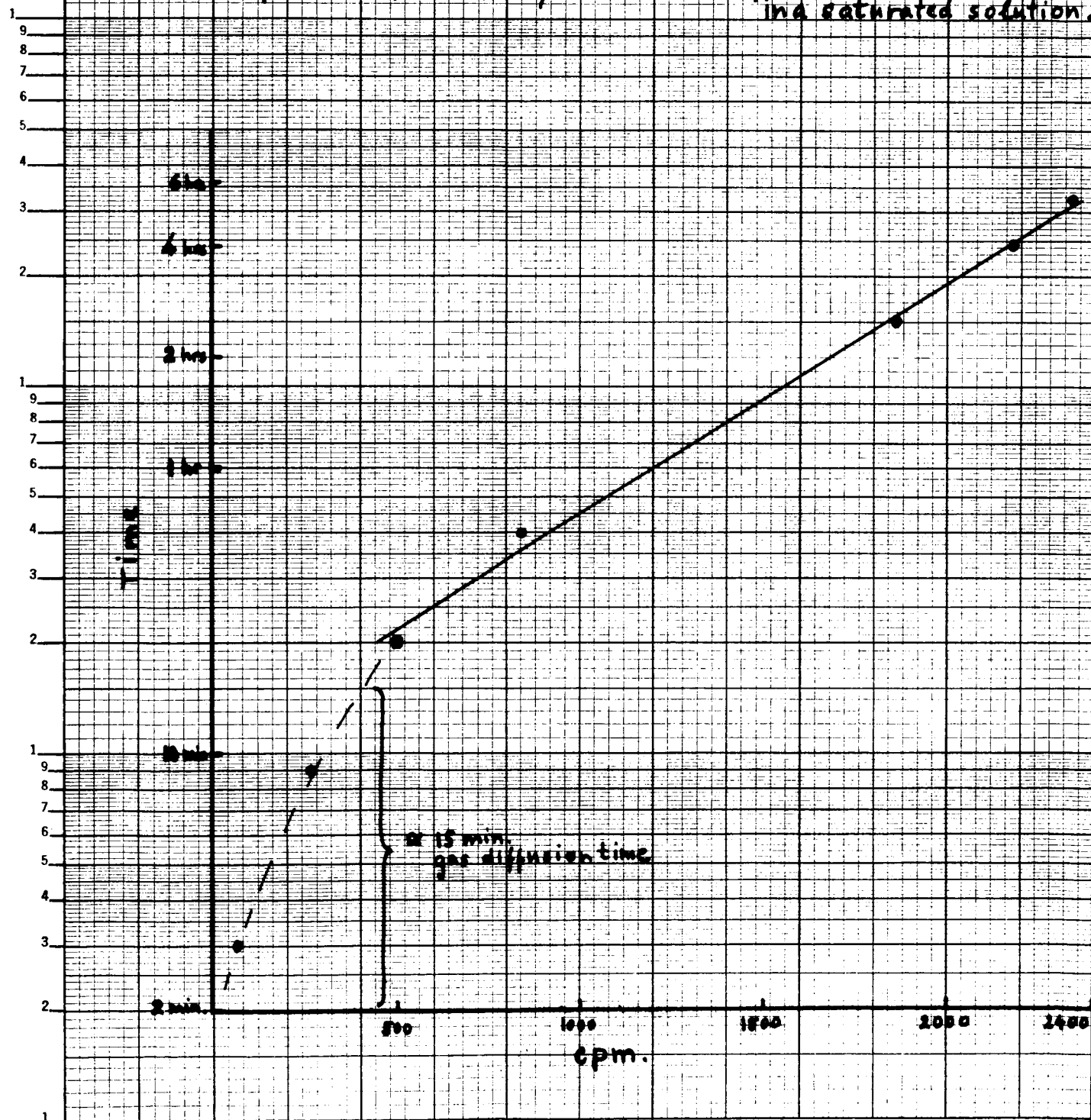


FIG. 7.

From: Howell, S.F., and Sumner, J.B.
The specific effects of buffers upon urease activity
J. Biol. Chem., 104, 619-626 (1934)

Urease activity vs. various buffers

0.1% urea, 0.125 M buffers

